Differential effects of saturated and unsaturated fatty acids on autophagy in pancreatic β-cells

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Abstract

Long chain saturated fatty acids are lipotoxic to pancreatic β-cells whereas most unsaturates are better tolerated and some may even be cytoprotective. Fatty acids alter autophagy in β-cells and there is increasing evidence that such alterations can impact directly on the regulation of viability. Accordingly, we have compared the effects of palmitate (C16:0) and palmitoleate (C16:1) on autophagy in cultured β-cells and human islets. Treatment of BRIN-BD11 β-cells with palmitate led to enhanced autophagic activity, as judged by cleavage of microtubule-associated protein 1 light chain 3—I (LC3-I) and this correlated with a marked loss of cell viability in the cells. In addition, transfection of these cells with an mCherry-YFP-LC3 reporter construct revealed the accumulation of autophagosomes in palmitate-treated cells, indicating an impairment of autophagosome-lysosome fusion. This was also seen upon addition of the vacuolar ATPase inhibitor, bafilomycin A1. Exposure of BRIN-BD11 cells to palmitoleate (C16:1) did not lead directly to changes in autophagic activity or flux but it antagonised the actions of palmitate. In parallel, palmitoleate also improved the viability of palmitate-treated BRIN-BD11 cells. Equivalent responses were observed in INS-1E cells and in isolated human islets. Taken together, these data suggest that palmitate may cause an impairment of autophagosome-lysosome fusion. These effects were not reproduced by palmitoleate which, instead, antagonised the responses mediated by palmitate suggesting that attenuation of β-cell stress may contribute to the improvement in cell viability caused by the mono-unsaturated fatty acid.

Introduction

Chronic exposure of pancreatic β-cells to long chain saturated fatty acids *in vitro* is associated with a phenomenon often referred to as "lipotoxicity" in which the cells display secretory dysfunction and, ultimately, an increased rate of apoptosis (Wang et al., 2019, Ruan et al., 2018, Ciregia et al., 2017, Mehmeti et al., 2017, Abdulkarim et al., 2017). By contrast, long chain mono- or poly-unsaturated fatty acids are much less toxic to β-cells and they can be overtly cytoprotective; even in the face of an otherwise lipotoxic stimulus (Liu et al., 2019b, Nemecz et al., 2018, Sramek et al., 2017, Sargsyan et al., 2016, Bellini et al., 2018). For example, incubation of β-cells *in vitro* with elevated concentrations of palmitate (C16:0) leads to loss of viability over a period of 18-36h, whereas treatment of these cells for the same period of time with a still higher total concentration of free fatty acid (FFA), yet comprising a mixture of palmitate plus palmitoleate (C16:1), is associated with the maintenance of cell viability ((Welters et al., 2004, Diakogiannaki et al., 2007, Dhayal et al., 2008). The molecular basis of the cytoprotective response seen under these conditions has not been defined fully but it has been reported that unsaturated FFAs attenuate some of the chronic stress responses associated with incubation of cells under more lipotoxic conditions. This has been studied principally in relation to the regulation of endoplasmic reticulum (ER) stress, where it has been shown that palmitoleate causes a reduction in the expression of pro-apoptotic transcription factors, such as ATF4 and CHOP, in cells treated with palmitate (Nemcova-Furstova et al., 2011, Green and Olson, 2011, Diakogiannaki et al., 2008).

ER stress is not the only mechanism involved in the regulation of cellular homeostasis in the face of environmental stressors and another important process which may become activated under these conditions is macroautophagy (hereafter termed "autophagy") (Assali et al., 2019, Varshney et al., 2017, Chen et al., 2017, Hu et al., 2016, Trudeau et al., 2016). Indeed, increasing evidence implies that ER stress and autophagy exist in a state of mutual counter-

balance to promote the maintenance of cell viability (Bugliani et al., 2019, Demirtas et al., 2016). Autophagy is a process by which the degradation and recycling of macronutrients is facilitated within cells and it involves the formation of double membrane bound vesicles ("autophagosomes") which ultimately enclose relevant substrates and fuse with lysosomes to mediate degradation (Eskelinen, 2019).

Several groups have reported that autophagy is affected when pancreatic β-cells are incubated with long chain saturated fatty acids but several conundrums remain unresolved. For example, some investigators have concluded that autophagy increases upon exposure of β-cells to palmitate whereas others report an impairment of autophagic flux under these conditions (Assali et al., 2019, Varshney et al., 2017, Hu et al., 2016, Sharma and Alonso, 2014). Less attention has been paid to the effects of long chain unsaturated fatty acids on autophagy but, again, a firm consensus is lacking. The response of cells to combinations of these molecules has been studied even less extensively. By way of illustration, Choi et al have provided evidence that exposure of β-cells to palmitate leads to alterations in autophagy and have suggested that this may occur as an early adaptive response designed to minimise the impact of lipotoxic stress (Choi et al., 2009). In support of this they, and others, noted that palmitate-mediated cytotoxicity was attenuated by rapamycin, an agent well known as an inducer of autophagy (Bugliani et al., 2019, Las et al., 2011, Bachar et al., 2009). A similar response was also reported recently in bone marrow mesenchymal cells (Liu et al., 2018). On this basis, it seems possible that regulation of autophagy may provide a mechanism by which the viability of β-cells can be influenced by environmental stressors and that saturated and unsaturated fatty acids may regulate autophagy differentially. In this context it is reported that additional cytoprotective agents such as spermidine and glucagon-like peptide-1 (GLP-1) can also regulate autophagy (Liu et al., 2019a, Zummo et al., 2017, Sharma et al., 2011, Tirupathi

Pichiah et al., 2011). Therefore, in the present work we have studied the effects of saturated and an unsaturated fatty acids on autophagy in two clonal rat β-cell lines, BRIN-BD11 and INS-1E cells, in which FFAs are known to influence viability differentially according to their degree of saturation.

Materials and Methods

Materials

RPMI-1640 medium, penicillin/streptomycin, glutamine, NuPAGE® Novex® Bis-Tris Gels and NuPAGE® MES running SDS buffer were from Invitrogen (Paisley, Scotland), foetal calf serum from PAA Laboratories (Yeovil, England); fatty acid-free bovine serum albumin and palmitoleate from MP Biomedicals (Thame, England). Docosahexanoic acid (DHA) and arachidonic acid (AA) were from Enzo Life Sciences (Exeter, England). Palmitate, myristate, laurate, the methyl esters of palmitate, palmitoleate, DHA, and AA; rapamycin, tunicamycin, bafilomycin A1 and antiserum against LC3 (L7543) were purchased from Sigma (Poole, England). PVDF membrane and Mini-Protean TGX gels were from Bio-Rad (Hemel Hempstead, England); stripping buffer (Re-Blot Plus-Strong) from Millipore. Antiserum against LC3 (ab58610) was purchased from Abcam (Cambridge, England) and other antibodies (phospho-mTOR (2971S); total mTOR (2972S), phospho S6K (9234S) & total S6K (9202S)), pEIF2α (3398P), total pEIF2α (5324P) were purchased from Cell Signalling (New England Biolabs) (Herts, England). Antiserum against GAPDH (5G4) was purchased from Hytest. pBABE-puro mCherry-EGFP-LC3B was a gift from Jayanta Debnath (25) (Addgene plasmid # 22418).

Cell culture, fatty acid treatment and viability analysis

BRIN-BD11 and INS-1E cell lines are derived from rat insulinoma by methods reported by McClenaghan et al (1996) and Merglen et al (2004). Both cell lines were cultured in RPMI-1640 medium containing 11 mM glucose supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 Uml^{-1} penicillin and 100 μgml^{-1} streptomycin. INS-1E culture medium was also supplemented with 50μM β-mercaptoethanol. For cell viability experiments, cells were seeded at a density of 0.5×10^5 cells/ml in complete medium for 24h. After this period, the medium was removed and replaced by serum free medium containing appropriate fatty acid-BSA complexes. Fatty acid stocks were prepared in ethanol and then bound to fatty acid free BSA by incubation at 37ºC for 1h. The final concentration of BSA used for cell incubations was maintained at 1% (w/v) and ethanol was 0.5% (v/v). Relevant vehicle (BSA plus ethanol) controls were included in each experiment. Cell death was quantified by flow cytometry after propidium iodide staining (Dhayal and Morgan, 2011) or by propidium iodide staining via fluorescence microscopy (Zummo et al., 2017).

Human Islets

Human islets were isolated from four non-diabetic donors at the Clinical Islet Lab, University of Alberta, Canada, or the Islet Isolation Unit, Kings College London, U.K., with appropriate ethical approval. Upon arrival, islets were maintained in CMRL media supplemented with 0.5% human albumin serum and 50 U/ml penicillin and 50 μg/ml streptomycin for 24 h prior to treatment with palmitate or palmitoleate as for the cell lines detailed above.

Western Blotting

BRIN-BD11 cells were seeded into $25cm^3$ flasks at a density of 2×10^6 cells/ml in complete medium for 24h and INS-1E cells were seeded into 12-well plates and cultured in complete

medium for 24h. The medium was then removed and replaced by serum free medium containing appropriate fatty acid-BSA complexes. After the required incubation period, whole cell protein was extracted and western blotting performed as described previously (Dhayal and Morgan, 2011) using antisera directed against LC3, phospho-mTOR, phospho-S6K, phospho-eIF2α, as described in individual experiments. For loading controls, membranes were stripped and probed for the presence of beta-actin, GAPDH, total mTOR, total S6K or total eIF2α.

Imaging using tandem fluorescent-tagged LC3 constructs (mCherry-EGFP-LC3 / mCherry-YFP-LC3)

BRIN-BD11 cells were seeded into fluorophore dishes at a density of $4x10^5$ cells/ml in complete medium for 24h. Thereafter, the cells were transiently transfected with an mCherry-YFP-LC3 construct. Culture medium was removed after 24h and replaced by serum free medium containing appropriate fatty acid-BSA complexes for a duration of 4h. Bafilomycin A1 was added one hour prior to the introduction of fatty acids. Images were captured with a Leica DMI8 confocal microscope using Leica Application Suite X software. Images were semi-automatically analysed using custom-built scripts in MATLAB in order to determine the number of LC3 puncta and level of autophagic flux per treatment.

For INS-1E, a stable cell line expressing mCherry-EGFP-LC3 was generated as previously described (Zummo et al., 2017). INS-1E (mCherry-EGFP-LC3) cells were plated on chambered coverslips and nuclei stained with 4.5 µg/ml Hoechst for 5 min. Live cell imaging was performed in a heated environmental chamber (37°C, 5% CO_2) using a Nikon TE2000 microscope (×100). Images were processed using NIS Element Imaging software and puncta quantified using Volocity software (Quorum Technologies).

Statistical analysis

Experiments were performed on either two or three separate occasions using either duplicates or triplicates for each experimental condition. Results are presented either as representative western blots or, for viability and autophagy analysis studies (n=3-6), as mean values ± SEM. For BRIN-BD11 cells, pairwise statistical comparisons are performed using a 2-sample t-test, whereas for INS-1E cells, values are compared by one or two-way ANOVA followed by a Bonferroni's test.

Results

Rapamycin improves cell viability and increases LC3-II accumulation in cultured β-cells

Initially, we sought to determine whether the anti-apoptotic effects of rapamycin reported previously in INS-1 cells and in foetal β-cells incubated under lipotoxic conditions (Choi et al., 2009, Las et al., 2011) are also observed in BRIN-BD11 cells. As expected, incubation of BRIN-BD11 cells with palmitate for 18h caused a dose-dependent loss of viability (Fig.1) while treatment with rapamycin alone did not lead to cell death over this time period. More specifically, the presence of rapamycin (up to 50ng/ml) was associated with a net improvement in cell viability. This was seen both under relatively benign conditions when BRIN-BD11 cells were incubated with vehicle alone (BSA plus ethanol; Fig.1) and also when cells were exposed to palmitate. To establish whether the cytoprotective action of rapamycin correlated with the induction of structural changes in proteins associated with autophagy in BRIN-BD11 cells, the cleavage of LC3-I was measured. Increased formation of LC3-II (from LC3-I) was observed in BRIN-BD11 cells within 15 minutes of exposure to rapamycin (Fig.2) and this response was sustained for at least 8h.

Palmitoleate improves cell viability and decreases LC3-II accumulation caused by palmitate

Given that rapamycin caused an improvement in cell viability during exposure of cells to palmitate, we next confirmed that palmitoleate protected BRIN-BD11 cells against the lipotoxic effects of palmitate (Fig.3a). Similar results were also observed in INS-1E cells (Fig 3b). However, unlike the situation with rapamycin, no increase in LC3-II generation was detected during 8h of exposure of BRIN-BD11 cells to palmitoleate (Fig.4a). By contrast, LC3-II was generated in cells exposed to palmitate alone (Fig.4a, 4b and 4c).

To investigate these phenomena further, the extent of LC3-II generation was examined in cells exposed to either palmitate or palmitoleate alone, or to the two FFAs in combination (Fig.4b and 4c). The data confirmed that palmitate caused a time-dependent increase in LC3-II formation (Fig. 4a-d) whereas palmitoleate did not. More strikingly, incubation of cells with 250μM palmitate and increasing concentrations of palmitoleate (Fig.4C) was associated with a reduction in palmitate-induced LC3-I cleavage. An equivalent reduction in LC3-II generation was not seen when cells were treated with the combination of palmitate and rapamycin (not shown), despite the fact that these incubation conditions were associated with an improvement in viability.

Likewise, in INS-1E cells, increased LC3-II generation was also observed in response to palmitate, whereas palmitoleate did not induce LC3-I cleavage and was able to inhibit the LC3-I cleavage induced by palmitate in these cells (Fig 5a). Most importantly, the results were replicated in human islets (Fig 5b). Culture of isolated human islets with 250μM palmitate in the presence of 20mM glucose increased LC3-I cleavage while co-incubation with 250μM palmitoleate attenuated this response (Fig 5b).

Generation of LC3-II reflects an increase in autophagic activity but, counter-intuitively, this can reflect either an increase or a decrease in net autophagic flux. Therefore, we examined the changes in autophagic flux more directly using dual reporter fluorescence constructs (mCherry-YFP-LC3 and mCherry-EGFP-LC3). Following expression of these constructs, an increase in the total number of red puncta is seen when autophagic activity is increased, whereas, an increase in the proportion of yellow puncta is observed under conditions when fusion of autophagosomes with lysosomes is inhibited. Both of these can be useful measures of autophagic flux, but the latter may be taken either as an indication of enhanced flux prior to lysosomal fusion or as evidence of blockage in the fusion mechanism. We have previously validated the quantification of red and yellow puncta as a measure of flux in INS-1E cells (Zummo et al., 2017). In the present study each of the constructs was expressed and the cells were then imaged following exposure to vehicle or fatty acids. Palmitate treatment increased the number of co-localised (yellow) puncta per cell (Fig. 6b), suggesting a potential impairment of autophagic flux, while concomitantly promoting overall autophagic activity (as judged by the total number of puncta; Fig 6b). By contrast, palmitoleate failed to elicit these effects. Indeed, palmitoleate decreased the number of yellow puncta found in cells exposed to palmitate and it also lowered the palmitate-induced increase in autophagic activity (total red puncta; Fig 6b). Palmitoleate similarly antagonised the effects of palmitate on autophagic flux (i.e. it reduced the number of yellow puncta) in INS-1 cells although treatment of these cells with palmitate did not increase the number of red puncta (Fig 7a,b).

Rapamycin and Palmitate induce LC3-I cleavage by different mechanisms

Examination of the time-course of palmitate-mediated LC3-II generation revealed that the response occurred much less rapidly than when cells were exposed to rapamycin (compare

Figs 2b & 4d) since a minimum of 4h exposure to palmitate was required before significant LC3 cleavage was observed (Fig.4d) whereas this occurred within a few minutes of addition of rapamycin (Fig.2b). This implies that the mechanisms involved in the actions of rapamycin and palmitate may be different; a proposition that was studied by examination of the extent of phosphorylation of mammalian target of rapamycin (mTOR) and ribosomal S6-kinase in response to each agent (Fig.8a). As its name implies, the protein kinase, mTOR, is a principal target for rapamycin and, consistent with this, its phosphorylation was reduced by the drug in BRIN-BD11 cells (Fig.8a). By contrast, palmitate failed to influence mTOR phosphorylation at any time point studied (Fig.8a). mTOR phosphorylation was similarly unaffected by the presence of palmitoleate (Fig.8b). Additionally, the extent of phosphorylation of the downstream mTOR substrate, S6K, was abolished during exposure of BRIN-BD11 cells to rapamycin (Fig.8a) but palmitate did not alter S6K phosphorylation.

Alterations in LC3-I cleavage by fatty acids mirrors their effects on cell viability

To establish whether the ability of long chain fatty acids to regulate β-cell viability correlates with their effects on autophagy, we next studied a range of structurally modified fatty acid derivatives having differential effects on viability. Exposure of BRIN-BD11 cells to the methyl-ester of palmitate failed to promote LC3-I cleavage (Fig 9a). In addition, two shorter chain saturated FFAs which are better tolerated than palmitate by BRIN-BD11 cells, myristate (C14:0) and laurate (C12:0) were also ineffective in this assay. It was also observed that the methyl-ester of palmitoleate failed to induce LC3-II generation but, intriguingly, in common with its unesterified parent fatty acid, the methyl-ester of palmitoleate prevented the cleavage of LC3-I seen in cells exposed to palmitate (Fig. 9b). The actions of two poly-unsaturated FFAs, arachidonic acid (C20:4) and docosahexaenoic acid (DHA; C22:6) as well as their respective methyl-esters, mirrored those of palmitoleate, in that, when added alone,

each failed to induce LC3-I cleavage but they markedly reduced the response in palmitate-treated cells (Fig. 9c).

Palmitoleate prevents the palmitate induced increase in LC3-1 cleavage by resolution of ER stress

We have previously demonstrated that palmitoleate can alleviate ER stress induced by palmitate in β-cells (Dhayal and Morgan, 2011, Diakogiannaki et al., 2008). Therefore, we also explored the effects of ER stressors on LC3 cleavage. As expected, tunicamycin, a known inducer of ER stress increased the phosphorylation of eIF2α in INS-1E and a similar response was seen with palmitate. Palmitoleate was able to alleviate this stress response in cells exposed to palmitate (Fig 10a) and palmitoleate also inhibited LC3-I cleavage induced by another ER stressor, tunicamycin (Fig 10b).

Discussion

Activation of autophagy is increasingly implicated in the regulation of cell viability, including in pancreatic β-cells, and a number of studies have suggested that alterations in autophagic activity occur in response to the addition of long chain fatty acids to these cells (Assali et al., 2019, Janikiewicz et al., 2015, Sharma and Alonso, 2014, Bugliani et al., 2019, Zummo et al., 2017). However, there is little consensus about the impact of fatty acids on autophagy in β-cells. Some reports suggest that palmitate induced toxicity is due to an increase in autophagic flux (Wu et al., 2017, Chen et al., 2013, Martino et al., 2012) whereas others suggest that palmitate impairs autophagic flux to induce cell death (RostamiRad et al., 2018, Liu et al., 2018, Xu et al., 2018, Hong et al., 2018). In the present work, we confirm

that autophagy is increased when cultured rodent β-cells are exposed to palmitate. We have employed two different approaches to reach these conclusions and our results imply that the loss of viability caused by palmitate in β-cells is due to an increase in cell stress responses associated with the changes in autophagic activity. This is evidenced by an increase in LC3-I cleavage (yielding LC3-II) monitored by Western blotting following exposure to palmitate and by quantification of the yellow puncta seen in cells transfected with dual reporter constructs (either mCherry-YFP-LC3 or mCherry-GFP-LC3) whose number was increased by palmitate. An increase in yellow puncta occurs when the rate of fusion between autophagosomes and lysosomes is attenuated, since successful fusion leads to quenching of the yellow fluorescence due to the acidic intraluminal environment of the resultant autolysophagosome. Thus, a rise in the number of yellow puncta implies a net reduction in flux through the pathway.

By contrast with palmitate, the long chain unsaturated fatty acid, palmitoleate, was inert in both assays of autophagy (LC3-I cleavage and changes in reporter fluorescence) suggesting that it does not directly alter the rate of autophagy in β-cells. Despite this, the presence of palmitoleate markedly affected the autophagic response to palmitate since, when the two fatty acids were present in combination, palmitate was much less effective at causing LC3-I cleavage and enhanced yellow puncta formation. Thus, although palmitoleate does not, itself, appear to influence the rate of autophagy directly in β-cells in the absence of palmitate, it clearly has the capacity to antagonise palmitate's ability to regulate autophagy.

Based on these observations, we were able to then consider, in more detail, the mechanisms by which palmitate and palmitoleate induce their differential effects on cell viability in β-cells and the extent to which changes in autophagy may contribute to these effects. To

address these issues, a range of additional reagents were employed. Initially, we compared the responses of β-cells to rapamycin and the two fatty acids since rapamycin is a well-known inducer of autophagy but it has also been reported to protect beta cells against cytotoxic stimuli. In this respect, its actions appear to mirror those of palmitoleate. However, as noted above, unlike rapamycin, palmitoleate failed to stimulate β-cell autophagy. Indeed, in the presence of palmitate, rapamycin protected the cells while increasing LC3-I cleavage whereas palmitoleate exerted its protective actions while simultaneously preventing LC3-I cleavage. Thus, there was a clear dissociation between the effects of rapamycin and palmitoleate on LC3-I cleavage in β-cells. There was an equally strong dissociation between rapamycin and palmitate with respect to the induction of autophagy since 18h exposure to rapamycin was associated with cytoprotection whereas, in the case of palmitate, cell death ensued. The simplest interpretation of these data is that a rise in autophagic activity is not directly cytotoxic to β-cells. This is consistent with emerging data in many cell types that autophagy is a process designed to maintain viability in the face of otherwise threatening environmental conditions.

At first sight, this conclusion does not, however, seem readily compatible with the present (and other) evidence indicating that an induction in autophagic activity occurs in β-cells exposed to palmitate, under conditions when cell viability is compromised. However, as noted above, use of dual reporter fluorescence constructs provided important information about the rates of autophagosome fusion with lysosomes in the distal phase of the autophagic pathway, in palmitate-treated cells. This revealed that, while palmitate causes a rise in autophagy in β-cells, the process does not go to completion but is interrupted by an impairment in the rate of autophagosome – lysosome fusion. By this means, palmitate causes

an increase in overall β-cell stress, as further evidenced by the enhancement of eIF2α phosphorylation seen under these conditions. Thus, it seems likely that the loss of viability deriving from exposure to palmitate occurs as a result of a cumulative increase in total cellular stress associated with impaired autophagic flux and dysfunctional ER responses. Electron microscopic analysis of palmitate-treated BRIN-BD11 cells supports this by revealing a dramatic dysregulation of ER architecture (Diakogiannaki et al., 2008).

Importantly, these cellular stress responses were minimised when cells were exposed simultaneously to both palmitate and palmitoleate. Thus, the cleavage of LC3-I was blocked under these conditions and the formation of yellow puncta was also attenuated in cells transfected with dual reporter constructs. Similarly, the palmitate-mediated increase in phosphorylation of eIF2α was also prevented by palmitoleate (as was the distension of ER seen under the electron microscope) (Dhayal and Morgan, 2011, Diakogiannaki et al., 2008). Indeed, we show here that the effects of another well-established ER stressor, tunicamycin, on eIF2α phosphorylation were also attenuated by palmitoleate. On this basis, we propose that the capacity of palmitoleate to sustain β-cell viability (despite the presence of an excess of palmitate or other stressors) relates to its ability to minimise the development of ER and autophagic stress responses. It may even be the case that palmitoleate can reverse stress responses after they have been initiated since it attenuates the toxic effects of palmitate even when introduced into the culture medium several hours after initial exposure of cells to the saturated fatty acid (Dhayal et al., 2008).

Consideration of these issues then raises a further important matter; namely the mechanism by which palmitoleate reduces β-cell stress and autophagy in palmitate-treated cells. The present work has not disclosed this mechanism in full but we have shown that a similar response is exerted both by other long-chain unsaturated fatty acids and by their equivalent

methyl-ester derivatives. This is important since the methyl-esters of long chain fatty acids are not activated by esterification to Co-enzyme A in β-cells and, as a consequence, they cannot be readily metabolised. This implies that their actions are likely to be mediated by more direct interaction with specific cellular targets in a manner equivalent to receptor activation (Morgan and Dhayal, 2010, Morgan and Dhayal, 2009). The identity of these putative molecular targets is still unknown but our results suggest that the quest to discover β-cell molecules that can mediate anti-apoptotic effects upon ligation of relevant unsaturated fatty acids is a worthwhile endeavour.

Declaration of interest

The authors declare no conflict of interest.

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Fig 1

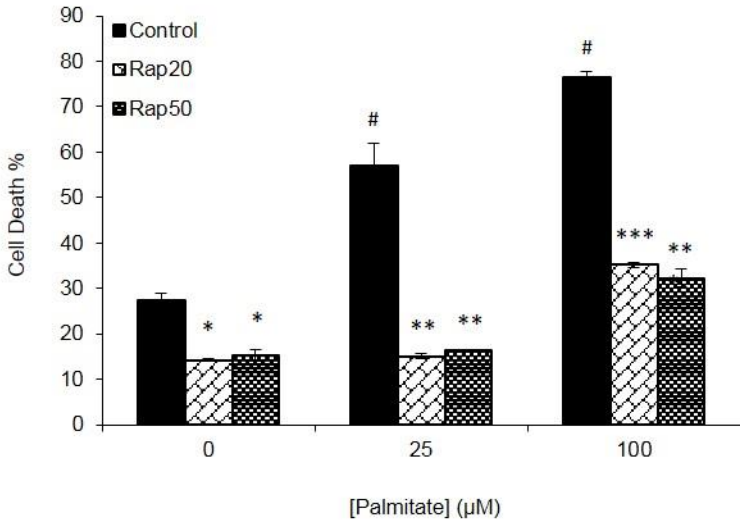


Figure 1: BRIN-BD11 cells were exposed to either 20ng/ml (Rap20) or 50ng/ml rapamycin (Rap50) in the absence or presence of 25μM or 100μM palmitate, as shown. Cell viability was assessed at the end of 18h incubation period by flow cytometry after staining with propidium iodide. *p<0.01 relative to control (0); #p<0.001 relative to control (0); **p<0.001 relative to 25μM palmitate alone; ***p<0.001 relative to 100μM palmitate alone.

Fig 2

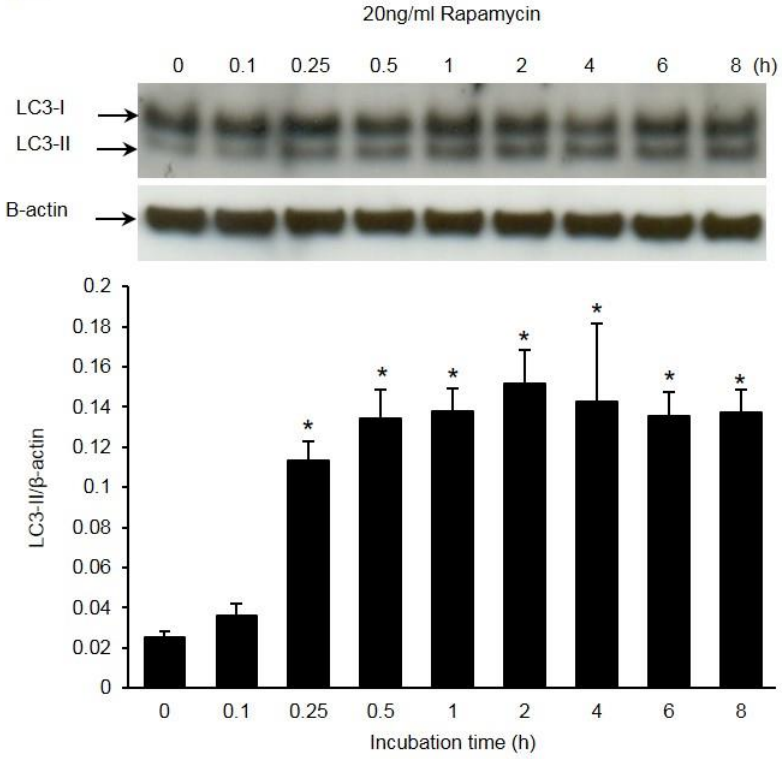


Figure 2: BRIN-BD11 cells were incubated with 20ng/ml rapamycin for increasing periods of time (up to 8h). Proteins were extracted from the cells and analysed by Western blotting using an antiserum directed against LC3. Beta actin was probed as a loading control and the bands were quantified by densitometry. *p<0.005 relative to condition 0h.

Fig 3a

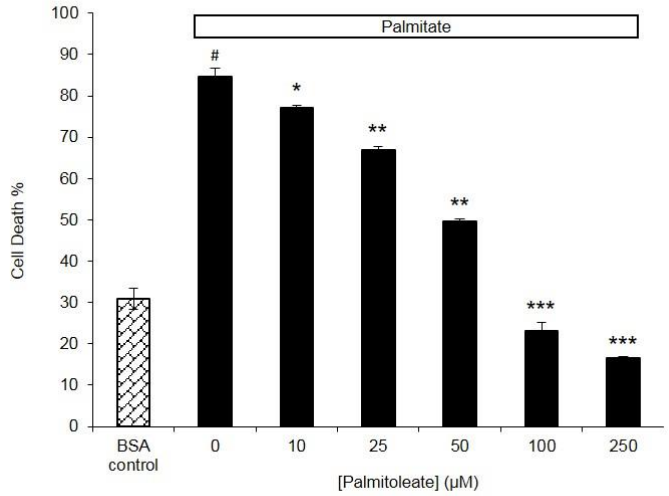


Fig 3b

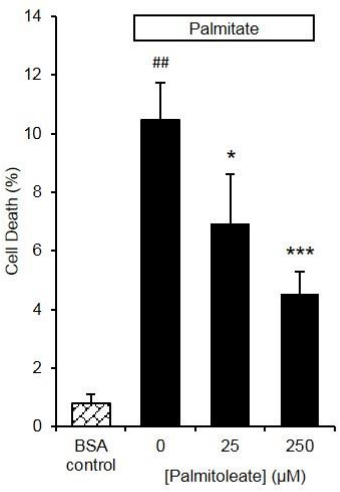


Figure 3a and b: **(a)** BRIN-BD11 cells were exposed to increasing concentrations of palmitoleate in the presence of 250µM palmitate for 18h. Cell viability was then assessed by flow cytometry after staining with propidium iodide. #p<0.001 relative to BSA control; *p<0.05 or **p<0.005 or ***p<0.001 relative to palmitate alone. **(b)** INS-1E cells were exposed to increasing concentrations of palmitoleate in the presence of 500µM palmitate for 18h. Cell viability was then assess by microscopic analysis after staining with propidium iodide. #p<0.05, ##p<0.01 relative to BSA control; *p<0.05 relative to palmitate alone.

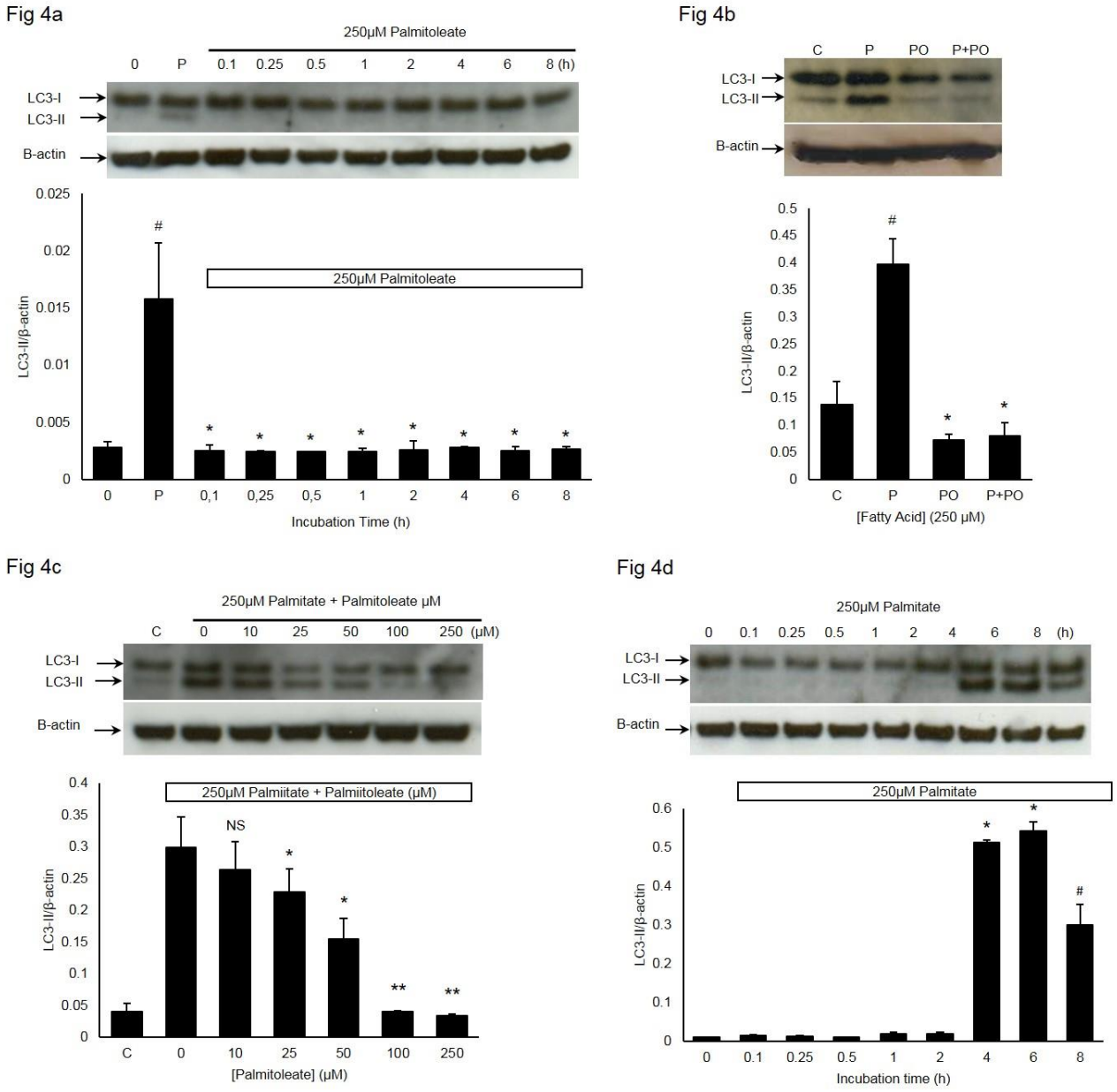


Figure 4a-d. BRIN-BD11 cells were incubated with **(a)** Either 250μM palmitate (P) for 4h or 250μM palmitoleate for increasing time periods (up to 8h) #p<0.05 relative to BSA control ; *p<0.05 relative to palmitate alone. ; or **(b)** Vehicle (C) or 250μM of palmitate (P) or palmitoleate (PO) either alone or in combination (P+PO) for 6h. #p<0.005 relative to BSA control; *p<0.005 relative to palmitate alone. Or **(c)** to increasing concentration of palmitoleate in the presence of 250μM palmitate for 6h. *p<0.01 and **p<0.001 relative to palmitate alone (0h). **(d)** 250μM palmitate for increasing time periods (up to 8h). *p<0.001 relative to condition at 0h and #p<0.005 relative to condition at 6h. Protein extracts were probed with an antiserum to LC3. Beta actin was used as a loading control and the bands were quantified by densitometry.

Fig 5a

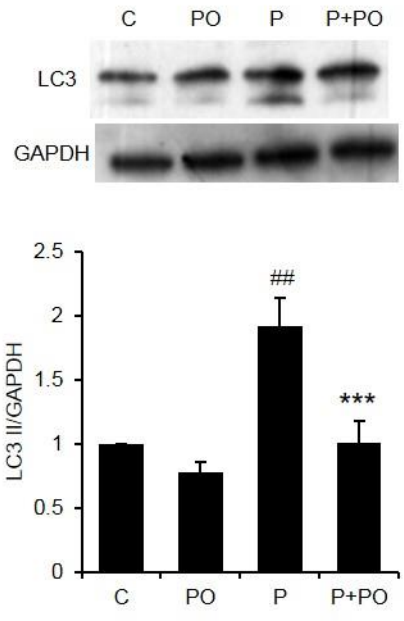


Fig 5b

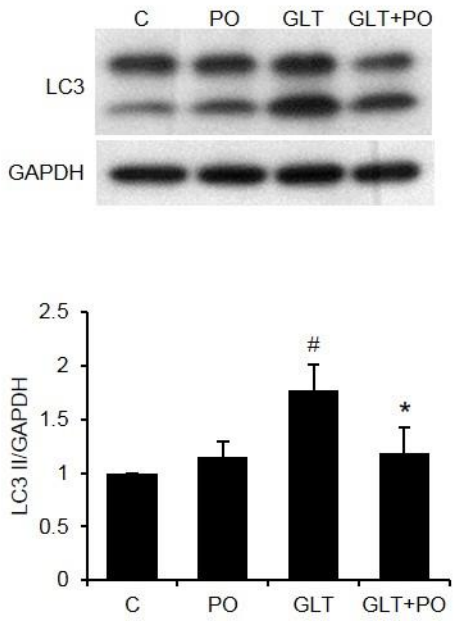
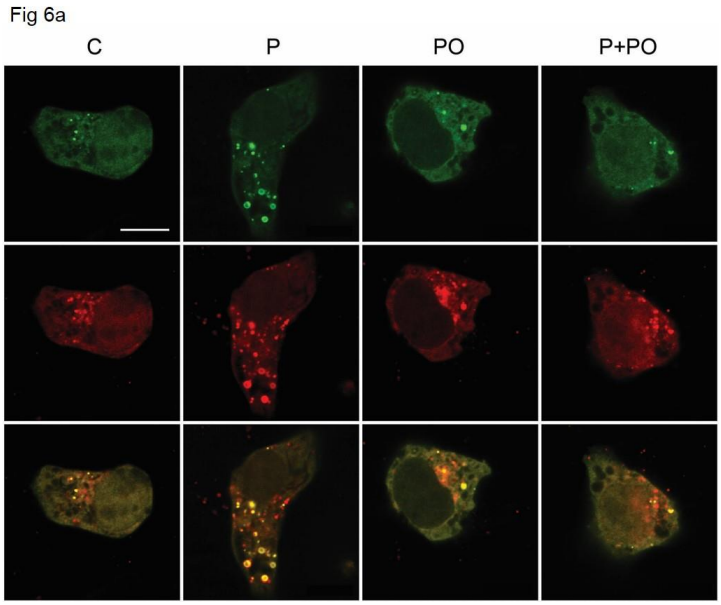


Figure 5a and b: (a) INS-1E were incubated with vehicle (C) or 250µM palmitoleate (PO) or 500µM of palmitate (P) either alone or in combination (P+PO) for 6h. **(b)** Human islets were incubated with vehicle (C) or palmitoleate (PO) or 25mM glucose containing 500µM of palmitate (GLT - glucolipotoxicity) either alone or with 250µM palmitoleate (GLT+PO) for 48h. Protein extracts were probed with an antiserum to LC3. GAPDH was used as a loading control and the bands were quantified by densitometry. Results normalised to control and are expressed as fold change. #p<0.05, ##p<0.01 relative to control; *p<0.05, **p<0.01 relative to palmitate alone.



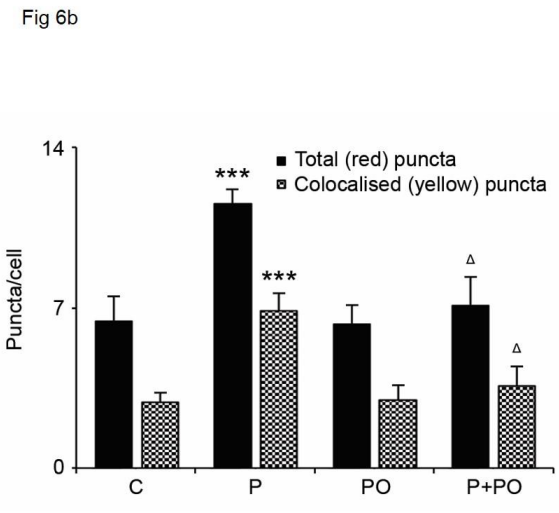


Figure 6a and b: (a) BRIN-BD11 cells expressing mCherry-YFP-LC3 construct were incubated with Vehicle (C), 250µM of palmitate (P), 250µM of palmitoleate (PO) or a combination of palmitate and palmitoleate (P+PO) for 4h. Cells were imaged using fluorescence microscopy and LC3-positive puncta counted using custom-built MATLAB scripts. Scale bar = 10µm. **(b)** Quantification of overall autophagic activity, measured by the total number of puncta per cell (red channel) and the number of colocalised puncta per cell (yellow channel). Represented are mean values ± SEM for the number of experiments (n=3). *p<0.05, **p<0.005 or ***p<0.001 for comparing FA treatments to their respective control treatments (C); $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.005 or $^{\Delta\Delta\Delta}$p<0.001 for comparing palmitate + palmitoleate (P+PO) treatments relative to their respective palmitate alone (P) treatments. All remaining pairwise tests show no significant differences.

Fig 7a

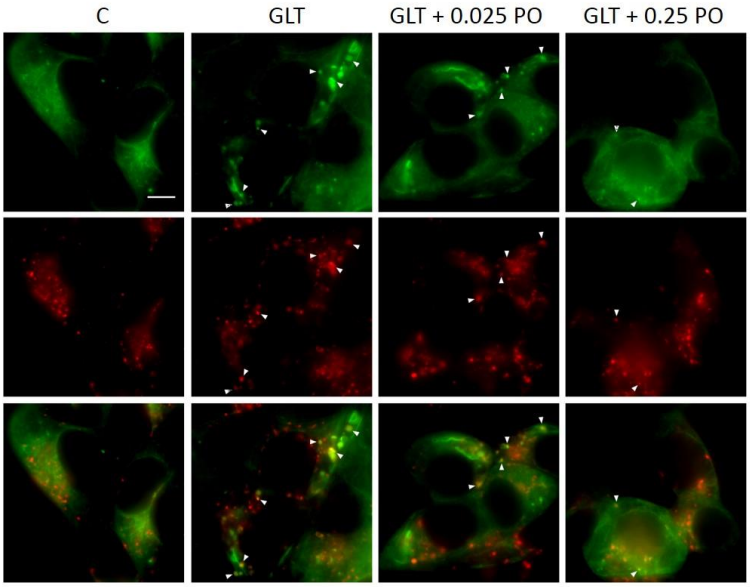


Fig 7b

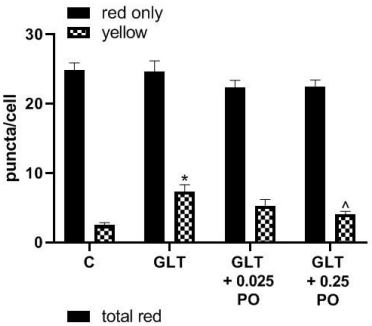


Fig 7c

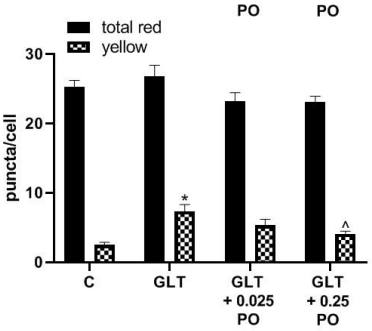


Figure 7a, b and c: (a) INS-1E cells expressing mCherry-GFP-LC3 construct were incubated with BSA control or 25mM glucose with 500μM palmitate (glucolipotoxicity - GLT) either alone or in combination with 25μM or 250μM palmitoleate for 4h. Cells were imaged using fluorescence microscopy and autophagic flux quantified by counting of red only and yellow puncta using Volocity software. White arrows indicate colocalising green and red (yellow) puncta. Scale bar = 10μm. **(b)** Quantification of autophagic flux, measured by the number of red only puncta per cell (red only) and the number of colocalised puncta per cell (yellow). **(c)** Cells were imaged using fluorescence microscopy and autophagic flux quantified by counting of total red and yellow puncta using Volocity software. Results are normalised to control. Represented are mean values ± SEM for the number of experiments (n=5). *$p<0.05$ for comparing FA treatments to their respective control treatments (C); $^{\Delta}$ $p<0.05$ for comparing GLT+PO treatments relative to their respective GLT alone treatments. All remaining pairwise tests show no significant differences.

Fig 8a

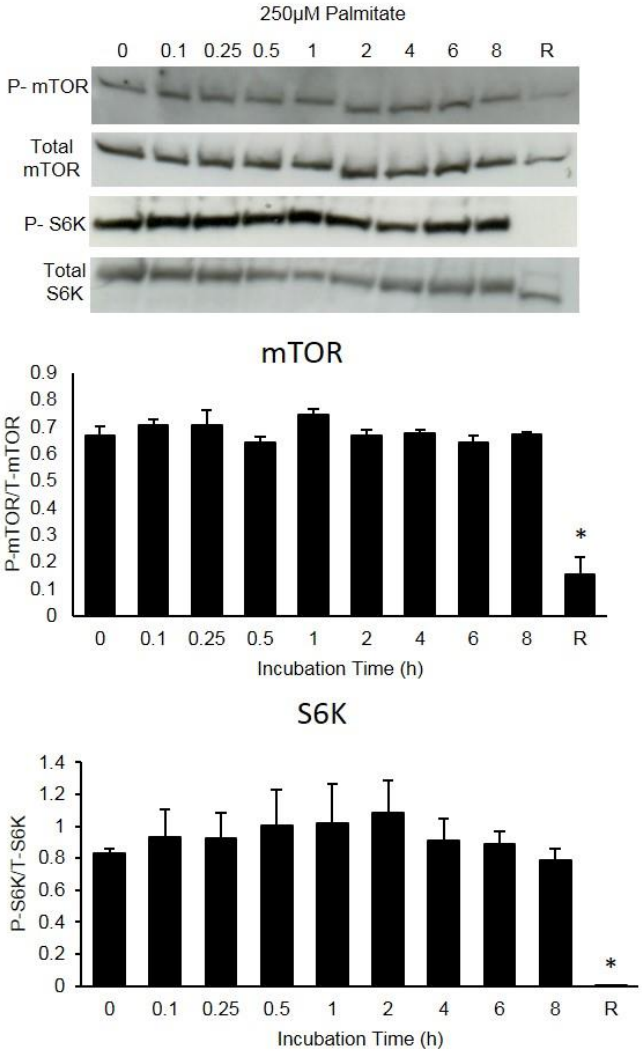


Fig 8b

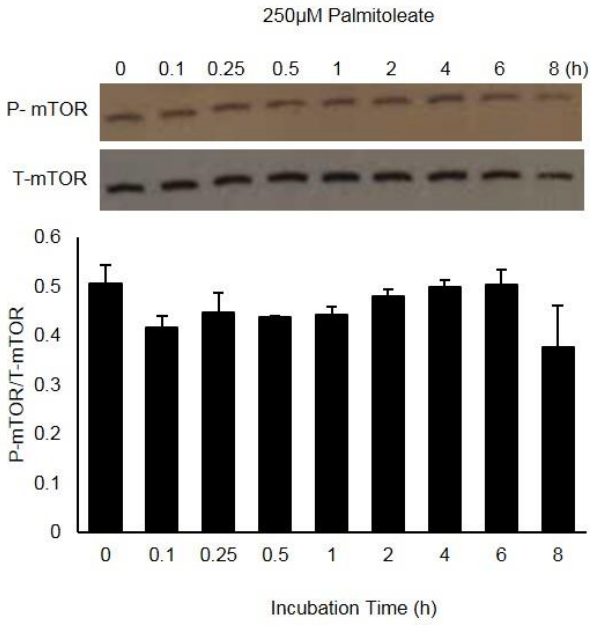


Figure 8a and b: BRIN-BD11 cells were exposed to **(a)** either 20ng/ml rapamycin (R) for 8h or 250μM palmitate for increasing time lengths (up to 8h). Protein extracts were probed with antisera to phospho mTOR (P-mTOR) and phospho S6K (P-S6K). *p<0.005 relative to condition 0h. Or **(b)** 250μM palmitoleate for increasing time lengths (up to 8h). Protein extracts were probed with antisera to phospho mTOR (P-mTOR). Total mTOR (T-mTOR) and total S6K (T-S6K) were used to demonstrate that changes in phosphorylated forms of the proteins were not due to alterations in the respective total protein levels within the cells.

Fig 9a

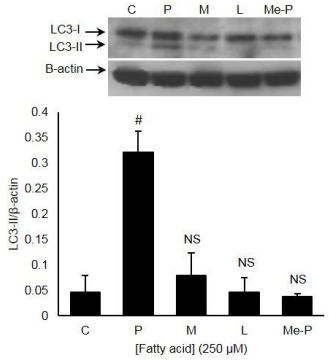


Fig 9b

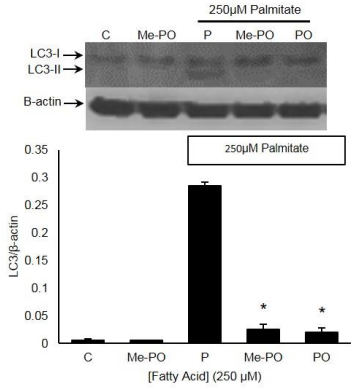


Fig 9c

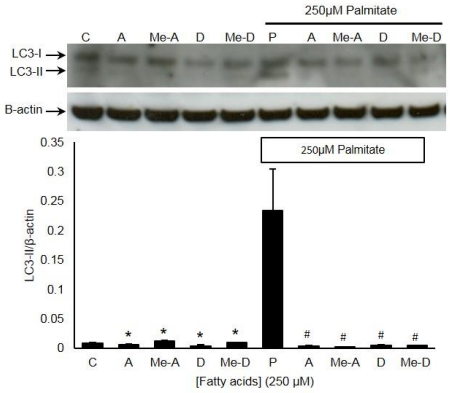


Figure 9a-c: BRIN-BD11cells were incubated under control conditions (C) or with **(a)** 250μM of each of palmitate (C16:0) (P), myristate (C14:0) (M), laurate (C12:0) (L) or the methyl-ester of palmitate (Me-P) for 6h. #p<0.005 relative to BSA control; ^{NS} non-significant relative to control. **(b)** 250μM palmitate (P) in the absence or presence of 250μM palmitoleate (P+PO) or methyl pamitoleate (P+Me-PO) for 6h. *p<0.005 relative to palmitate. **(c)** 250μM of each of arachidonate (A), arachidonate methyl ester (Me-A), DHA (D) or methyl-DHA (Me-D) either alone or in the presence of palmitate (P) for 6h. #p<0.005 relative to BSA control; *p<0.005 relative to palmitate. Protein extracts were probed with an antiserum to LC3. Beta actin was used as a loading control and the bands were quantified by densitometry.

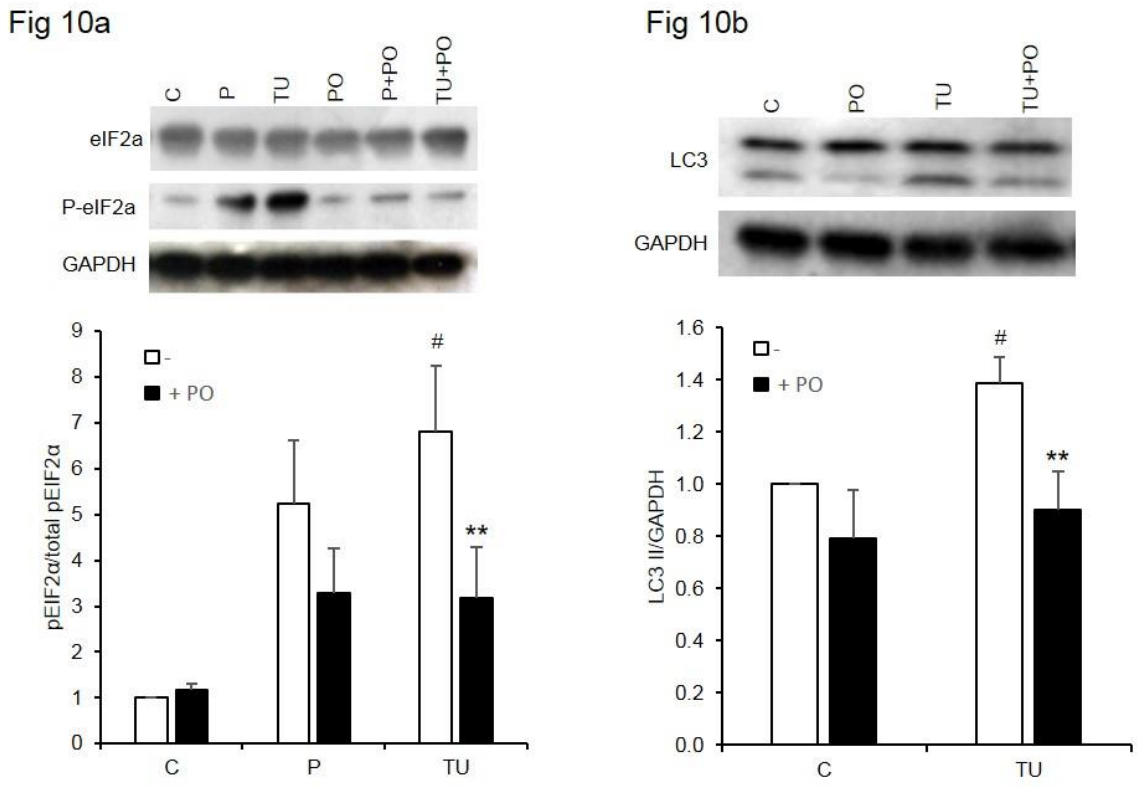


Figure 10a and b: INS-1E were incubated with Vehicle (C), 500µM of palmitate (P) or 5 µg/ml tunicamycin (TU) with or without 250µM palmitoleate (PO) for 18 h. Protein extracts were probed with an antiserum to p-eIF2α and total eIF2α **(a)** or LC3 **(b)**. GAPDH was used as a loading control and the bands were quantified by densitometry. Results normalised to control and are expressed as fold change. #p<0.05, ##P<0.01 relative to control; **p<0.01 relative to palmitate alone.